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Evolution of Developmental Control Mechanisms

A novel N-terminal motif is responsible for the evolution of neural crest-specific gene-regulatory activity in vertebrate FoxD3

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ABSTRACT

The neural crest is unique to vertebrates and has allowed the evolution of their complicated craniofacial structures. During vertebrate evolution, the acquisition of the neural crest must have been accompanied by the emergence of a new gene regulatory network (GRN). Here, to investigate the role of protein evolution in the emergence of the neural crest GRN, we examined the neural crest cell (NCC) differentiation-inducing activity of chordate *FoxD* genes. Amphioxus and vertebrate (*Xenopus*) FoxD proteins both exhibited transcriptional repressor activity in Gal4 transactivation assays and bound to similar DNA sequences *in vitro*. However, whereas vertebrate *FoxD3* genes induced the differentiation of ectopic NCCs when overexpressed in chick neural tube, neither amphioxus *FoxD* nor any other vertebrate *FoxD* paralogs exhibited this activity. Experiments using chimeric proteins showed that the N-terminal portion of the vertebrate FoxD3 protein is critical to its NCC differentiation-inducing activity. Furthermore, replacement of the N-terminus of amphioxus FoxD with a 39-amino-acid segment from zebrafish FoxD3 conferred neural crest-inducing activity on amphioxus FoxD or zebrafish FoxD1. Therefore, fixation of this N-terminal amino acid sequence may have been crucial in the evolutionary recruitment of FoxD3 to the vertebrate neural crest GRN.

Introduction

One of the major challenges of evolutionary developmental biology is elucidating how novel structures can be created solely through changes in genetic information. It is generally accepted that the morphologic features of various multicellular animals arise from a common set of "toolkit" genes (Carroll et al., 2001). This premise inevitably implies that novel features emerged primarily as a result of alterations in gene expression patterns, and that these altered patterns of gene expression were brought about by the evolution of *cis*-regulatory elements, as proposed by Carroll et al. (2001) and Davidson (2006). However, Kawashima et al. (2009) have pointed out that novel genes produced by domain shuffling may also play a critical role in the evolution of novel structures. They showed that genes acquired in the common ancestors of chordates are involved in the development of their characteristic features. In the common ancestors of the vertebrates, for example, the genes encoding Aggrecan, Occludin, and Tectorin alpha were built up by domain shuffling and were perhaps involved in the evolution of cartilage, tight junctions, and tectorial membranes, respectively (Kawashima et al., 2009).

Novel sequence motifs in transcription factors have also been implicated in the evolution of morphologic features. For example, the glutamine–alanine-rich sequence (QA domain) of insect Ubx is thought to have been important in the evolutionary loss of abdominal appendages (Galant and Carroll, 2002; Ronshaugen et al., 2002). Similarly, the N-terminal motif of the *Daphnia* Antennapedia protein has also been implicated in the evolution of their specific appendage morphology (Shiga et al., 2002). Lynch et al. (2008) presented evidence that modification to HoxA-11 was essential in the evolution of mammalian pregnancy, as the modified protein has acquired a novel regulatory relationship with the prolactin gene. These studies have revealed that the evolution of morphology is driven not only by the molecular evolution of *cis*-regulatory elements but also by the evolution of protein coding sequences.

Neural crest cells (NCCs) first arose in the ancestors of vertebrates and have performed a central role in the evolution of vertebrates, particularly in their complicated craniofacial structures (Gans and Northcutt, 1983). The gene regulatory network (GRN) underlying NCC differentiation has been intensively studied. The transcription factor genes known as "neural plate border

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specifiers", including *zic*, *Pax3/7*, *Dlx* and *msx*, regulate the regional differentiation of the boundary between the neural and non-neural ectoderm, from which the NCCs emerge (Meulemans and Bronner-Fraser, 2004). The transcription factor genes, known as "neural crest specifiers", including *slug/snail*, *Foxd3*, *AP-2*, *Sox9/10* and *Twist* act downstream of the neural plate border specifiers, regulating the differentiation of NCCs by controlling the expression of neural crest effectors, such as cadherins and collagens (Meulemans and Bronner-Fraser, 2004). Notably, in protochordates (both amphioxus and ascidians), homologs of the neural plate border specifiers are expressed in the border region between the neural and epidermal ectoderm (Holland et al., 1996; Wada et al., 1997; Aniello et al., 1999; Holland et al., 1999; Sharman et al., 1999; Caracciolo et al., 2000; Gostling and Shimeld, 2003; Meulemans and Bronner-Fraser, 2004; Wada and Makabe, 2006; Yu et al., 2008). In contrast, homologs of the neural crest specifiers (with the exception of *snail/slug*) are not expressed in the corresponding regions of protochordates; thus, the neural crest specifiers are likely to be new recruits to the neural crest GRN (Langeland et al., 1998; Imai et al., 2002; Meulemans and Bronner-Fraser, 2002, 2004; Yu et al., 2004, 2008; Wada and Makabe, 2006; Meulemans and Bronner-Fraser, 2007; Wada, 2010). It has been proposed that by co-opting neural crest specifier genes into a pre-existing neural plate border specification genetic network during early vertebrate evolution, cells at the neural plate border region acquired new cellular properties, such as migration and the ability to differentiate into diverse cell types, and evolved into neural crest cells (Meulemans and Bronner-Fraser, 2004, 2005; Yu, 2010). This idea is supported by recent experiments in ascidians showing that ectopic expression of homolog of one of the neural crest specifier genes (*Twist*) can reprogram neural plate border-derived pigment cells into migratory mesenchymal cells (Abitua et al., 2012).

During this process of co-option, some transcription factors may have continued to regulate the same downstream genes that they regulated in the ancestral context, only now also in NCCs. In addition, they may have acquired new target genes, possibly by gaining the ability to physically interact with other transcription factors. This process would have activated new target genes in the NCCs that were not activated in the ancestral context. Thus, we reason that neofunctionalization of transcription factors might be accompanied by the evolutionary fixation of new sequence motifs, particularly those involved in intermolecular interactions.

In the present study, we focused on the transcription factor FoxD3 (Forkhead box D3). Because two rounds of genome duplication occurred during the evolution of vertebrates (Putnam et al., 2008), most vertebrate neural crest specifiers have several paralogs in vertebrate species but only a single homolog in protochordate species (reviewed in Wada and Makabe, 2006). For some other neural crest specifiers, including *Sox9/10*, *snail/slug*, and *AP-2*, duplicate paralogs are expressed in vertebrate NCCs (Hilger-Eversheim et al., 2000; Linker et al., 2000; Hong and Saint-Jeannet, 2005), indicating that co-option of these genes occurred before the genome duplications. In contrast, among five known vertebrate paralogs of *FoxD*, only *FoxD3* is expressed in the neural crest; the other paralogs have retained their ancestral chordate roles in the forebrain, somites, and notochord (Kos et al., 2001; Sasai et al., 2001; Yu et al., 2002; Yu, 2010). Therefore, we decided to focus on FoxD3 in our attempts to detect specific amino acid sequences involved in the neofunctionalization of FoxD underlying neural crest specification.

In the present study, we examined the molecular evolution underlying the neofunctionalization of FoxD3 by examining the NCC differentiation-inducing activity of genes of the *FoxD* family in vertebrates and amphioxus, the most basal group of chordates (Bourlat et al., 2006; Putnam et al., 2008). We found that amphioxus and vertebrate FoxD proteins both function as transcriptional repressors, binding to similar DNA sequence motifs. However, when overexpressed in chick neural tubes, only vertebrate *FoxD3* induces the production of ectopic NCCs; neither amphioxus *FoxD* nor any other vertebrate *FoxD3* paralogs (such as *FoxD1* or *FoxD5*) exhibit this activity. Furthermore, by assaying the activity of chimeric FoxD proteins, we identified the N-terminal region of the FoxD3 protein as the essential region for ectopic induction of NCCs. These results indicate that the involvement of FoxD3 in the GRN of NCC differentiation was accompanied by fixation of the N-terminal sequence motif. Our findings constitute the first evidence linking the evolution of vertebrate NCCs to the molecular evolution of a specific protein sequence.

Materials and methods

Cloning of expression vectors

The Gal4-responsive luciferase reporter gene construct containing five Gal4-binding sites and expression vectors encoding the DNA-binding domain of Gal4 fused to a hemagglutinin tag (pGalHA) or to a mouse Pax6 transactivation domain (pGal-mPax6TA) or glutathione S-transferase (GST) fused to the Groucho corepressor protein Grg4 (pGST-Grg4) have been described previously (Czerny and Busslinger, 1995; Kozmik et al., 2003). The complete open reading frames of the amphioxus *FoxD* gene (*AmphiFoxD*) and *Xenopus FoxD1*, *FoxD3*, and *FoxD5* (*xFoxD1*, *xFoxD3*, and *xFoxD5*, respectively) were amplified from cDNA by PCR, and cloned into pGalHA and pCS2+. Coding sequences for AmphiFoxD deletion constructs were generated by PCR and cloned into pGalHA. The AmphiFoxD octapeptide mutation F311E was generated using a QuickChange mutagenesis kit (Stratagene).

Plasmid constructs for chick electroporation were made using the pCAGGS vector (Momose et al., 1999). The complete open reading frames of FoxD genes from *Xenopus*, zebrafish, mouse and amphioxus were inserted into pCAGGS. Chimeric protein constructs were produced by amplifying partial cDNA fragments and inserting them into pCAGGS. The sequences of the chimeric constructs are shown in Supplementary Figs. 1–6, respectively. We confirmed that no mutation occurred during plasmid construction by sequencing.

Cell transfection, electrophoretic mobility shift assays (EMSAs), and transactivation assays

Cells of the African green monkey kidney fibroblast cell line COS-7 were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (PAA Laboratories), 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The cells were transfected with pCS-FoxD expression vectors, cultured for 48 h, and then harvested. Nuclear extracts were made from the cells and used in EMSAs with double-stranded DNAs containing various FoxD DNA-binding sites. The upper-strand sequences of the oligonucleotides used for EMSAs were (5′–3′): FoxC1(wt), gatccaaagtaaataaacaacaga; FoxC1(mut), gatccaaagtaaattaacaacaga; FoxD1(siteB), gatcccttaagtaaacaaacagagatc; FoxD1(siteE), gatccaggccgtaaacaaacagagatc; FoxD1(mut), gatcccttaagtacccaaacagagatc; FoxD2, aattcgactgcttaagtaaacaatgggccctcgtgcat; FoxD3, gcttaaaataacaatac; and Pax6HD (negative control), tcgagcatcaggatgctaattggattagcatccgatcgg.

Double-stranded oligonucleotides containing the indicated FoxD binding sites were radioactively labeled at their 5′ ends with [γ^{32}P]-dATP using polynucleotide kinase (Boehringer Manheim) and purified on microspin columns (Amersham Biosciences). The labeled oligonucleotides were incubated with COS-7-derived

AmphiFoxD, xFoxD1, or xFoxD3 protein in binding reactions consisting of 4% Ficol, 10 mM Tris–HCl (pH 8), 1 mM dithiothreitol, 1 mM EDTA, 100 mM KCl, and 50 μg/ml poly(dI–dC).

For luciferase reporter gene transactivation assays, COS-7 cells were transiently cotransfected with a plasmid harboring a Gal4-responsive firefly luciferase-based reporter gene, an internal control plasmid for transfection efficiency (*pCMV-lacZ*), and a FoxD expression vector using FuGENE6 (Roche Molecular Biochemicals). The total amount of transfected plasmid DNA was 300 ng/well of a 24-well plate. Two days later, reporter activity was assayed using a luciferase assay kit (Promega). Expression of the *pCMV-lacZ* reporter in transfected cells was measured using a β-galactosidase luminescence kit (Galacto-Star; Applied Biosystems) and used to normalize the data for transfection efficiency. Transfection experiments were performed in triplicate; data shown represent means of triplicates ± standard deviation.

GST pull-down assays

FoxD and luciferase proteins labeled with ^{35}S were prepared *in vitro* from pCS-based expression vectors using TNT Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer's protocol. GST and GST-Grg4 expression plasmids were transformed into *E. coli* BL21 CodonPlus (DE3)-RIPL cells (Stratagene), and the corresponding expressed proteins were mixed with glutathione–Sepharose beads (BD Bioscience). The beads were washed three times with 5 ml of binding buffer (20 mM Tris pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 0.1% Nonidet P-40), and the proteins remaining bound to the beads were analyzed by SDS-PAGE. Beads containing normalized amounts of fusion protein were blocked in binding buffer containing 5 mg/ml of bovine serum albumin (BSA) for 2 h at 4 °C and resuspended in binding buffer containing 1 mg/ml BSA and 100 μg/ml ethidium bromide. The beads were incubated overnight at 4 °C with *in vitro*-translated ^{35}S-labeled FoxD protein or luciferase (negative control). The beads were washed three times with binding buffer and boiled with SDS sample buffer. The released interacting proteins were separated by SDS-PAGE and detected by autoradiography.

Electroporation of plasmid DNA into chick neural tubes

Plasmid DNA was electroporated into chick neural tubes essentially as described in Wada et al. (2006). Circular plasmid DNA (3 mg/ml) was injected into the neural tube lumen of chick embryos at Hamburger–Hamilton (HH) stage 09 at the level of the trunk, and five square pulses of 20 mV were applied for 50 msec each. Embryos were fixed 24 h after electroporation (at HH stage 20–22) for staining. In order to visualize efficiency of electroporation, GFP expression vector (pCAGGS-GFP; Wada et al., 2006) was co-electroporated.

Immunohistology and in situ hybridization

After electroporation, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C for 40 h, transferred through a methanol/PBS gradient, and stored in 100% methanol at −20 °C until use. Specimens were sectioned after frozen in O.C.T. (Optimal Cutting Temperature) compounds by using CM3050 III (Leica). *In situ* hybridization was performed on sectioned specimens following Wada et al. (2006). Immunohistochemical analysis was performed with monoclonal antibody of HNK-1 (mouse IgM, Tucker et al., 1988), and polyclonal antibody against GFP (Clonetech).

Results

FoxD proteins from both amphioxus and vertebrates have transcriptional repressor activity and interact with the Groucho corepressor Grg4

To compare the basic transcriptional properties of amphioxus FoxD (AmphiFoxD) with those of its vertebrate cognates, we used a Gal4 transactivation assay. A plasmid encoding Gal4 (negative control), Gal4 fused to a mouse Pax6 transactivation domain (Gal4–Pax6; positive control), or Gal4 fused to AmphiFoxD or *Xenopus* FoxD1, FoxD3, or FoxD5 (xFoxD1, xFoxD3, and xFoxD5, respectively) was cotransfected into COS-7 African green monkey kidney cells with a Gal4-responsive luciferase-reporter plasmid. As expected, the Gal4–Pax6 fusion protein activated transcription of the luciferase reporter gene. All of the Gal4–FoxD fusion proteins repressed transcription of the reporter gene with similar potency (Fig. 1A), indicating that the amphioxus and vertebrate FoxD proteins act as transcriptional repressors.

To identify the functional domains within AmphiFoxD that mediate transcriptional activity, Gal4 fusion constructs expressing different regions of AmphiFoxD were cotransfected into COS-7 cells together with the Gal4 reporter plasmid. The Gal4 fusion proteins containing amino acids 1–110, 110–210, 210–318, or 311–402 of AmphiFoxD exhibited transcriptional repressor activity (Fig. 1B), suggesting that AmphiFoxD contains several repressor domains.

A closer examination of the AmphiFoxD sequence revealed that amino acids 311–318 of AmphiFoxD constitute the conserved octapeptide sequence FSIENIIG. This sequence, sometimes called the engrailed homology (EH) motif, is often found in transcriptional repressor proteins. Therefore, we created a modified the Gal (210–318) construct to remove the octapeptide sequence. The resulting Gal(210–311) construct failed to repress transcription when cotransfected into COS-7 cells with the Gal4 reporter plasmid (Fig. 1B), suggesting that the EH octapeptide functions as a repressor domain in AmphiFoxD. To confirm this hypothesis, we altered the Gal(311–402) construct to mutate the conserved phenylalanine residue within the EH octapeptide (AmphiFoxD residue 311) to glutamate. As shown in Fig. 1B, this change decreased the transcriptional repressor activity of the resulting Gal(311–402)FE construct, suggesting that the conserved EH octapeptide region at 311–318 mediates repression.

The presence of the conserved EH octapeptide prompted us to investigate the possibility of a direct interaction between FoxD proteins and Groucho corepressor proteins. The Groucho/Grg corepressors are known to interact with EH octapeptide sequences in many DNA binding proteins (Fisher and Caudy, 1998; Jennings et al., 2006). To examine whether FoxD proteins interact directly with Grg4, we performed GST pull-down assays with a GST–Grg4 fusion protein. The fusion protein pulled down *in vitro*-translated AmphiFoxD, xFoxD1, xFoxD3, and xFoxD5, but not the negative control (luciferase) protein (Fig. 1C). In contrast, GST alone was unable to interact with FoxD proteins. These results suggest that both the amphioxus and vertebrate FoxD proteins interact directly with the Grg4 corepressor.

Amphioxus and vertebrate FoxD proteins share similar DNA binding specificity

Previously reported EMSA experiments have shown that Fox proteins bind multiple target sites *in vitro* (Pierrou et al., 1994; Wu et al., 1998; Jin et al., 1999; Saleem et al., 2001). Although the consensus DNA sequence bound by FoxD proteins is not well defined, all FoxD-binding DNA sequences described thus far are AT-rich. Therefore, to examine whether AmphiFoxD has the same DNA binding specificity as vertebrate FoxD (represented by xFoxD1 and xFoxD3), we performed EMSA experiments with a

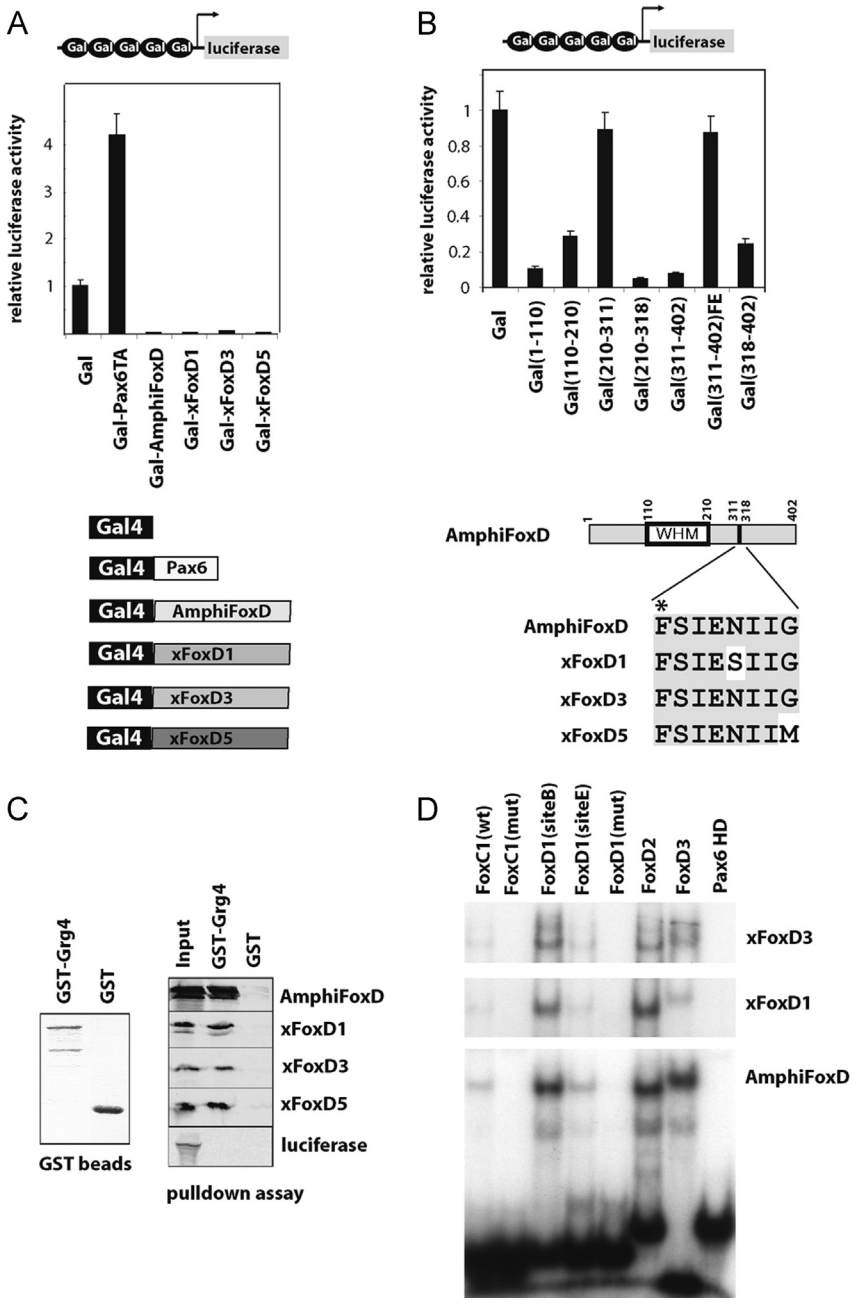

Fig. 1. Functional similarity between amphioxus and vertebrate FoxD transcription factors. (A) FoxD proteins are transcriptional repressors. Plasmid constructs encoding the Gal4 DNA-binding domain fused to FoxD (lower) and the Gal4-responsive luciferase reporter gene were cotransfected into COS-7 cells. A β-galactosidase expression plasmid was also cotransfected to allow normalization for transfection efficiency. Luciferase reporter assays were performed as described in the "Materials and methods" section. (B) Structure–function analysis of the transactivation properties of amphioxus FoxD (AmphiFoxD). The indicated regions of AmphiFoxD were tested in the Gal4 transactivation assay. The octapeptide-like sequence represents a repressor domain conserved within the FoxD family. (C) AmphiFoxD interacts with the Grg4 corepressor. A glutathione S-transferase (GST) pull-down assay was performed using GST and GST–Grg4. The normalized amounts of the GST proteins used in the pull-down assay are shown in the Coomassie blue-stained gel in the left panel. The *in vitro*-translated ^{35}S-labeled FoxD proteins or negative-control luciferase protein were incubated with the indicated GST fusion proteins bound to glutathione beads. Interacting proteins were detected by autoradiography (right panel). (D) An electrophoretic mobility shift assay (EMSA) showing that the DNA binding sequence specificities of AmphiFoxD and the *Xenopus* FoxD proteins xFoxD1 and xFoxD3 are similar *in vitro*.

panel of oligonucleotides (shown in the *Materials and methods* section) representing previously defined DNA-binding sites for FoxC1, FoxD1, FoxD2, and FoxD3 (Pierrou et al., 1994; Wu et al., 1998; Jin et al., 1999; Saleem et al., 2001). As shown in Fig. 1D, each FoxD protein tested, including AmphiFoxD, recognized the target sites with similar affinities and were similarly sensitive to mutations in the target sites. When an AT-rich Pax6 homeodomain target sequence (Czerny and Busslinger, 1995) was used as a control for non-specific binding, no mobility shift occurred for any of the FoxD proteins. These results suggest that the DNA binding specificity of AmphiFoxD is very similar, if not identical, to that of vertebrate (*Xenopus*) FoxD1 and FoxD3.

Overexpression of vertebrate FoxD1, FoxD2, FoxD4, FoxD5, and amphioxus AmphiFoxD do not induce ectopic NCC differentiation in chick embryo

During vertebrate evolution, each FoxD paralog acquired a specific function (Yu et al., 2002). This specialization probably arose through the acquisition of distinct sets of target genes for

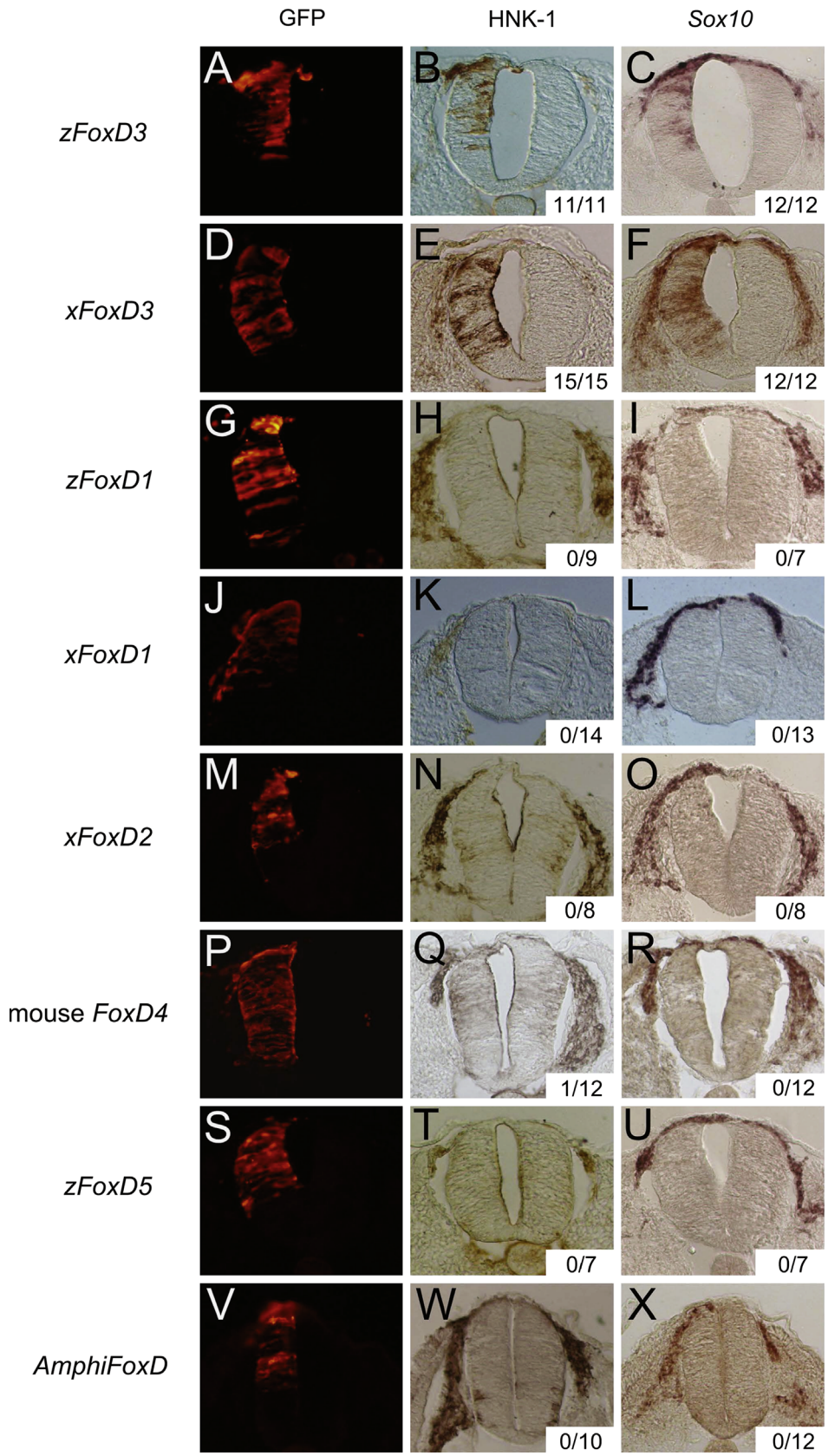

Fig. 2. Effect of FoxD overexpression on HNK-1 antigen and *Sox10* expressions in chick neural tube. Upregulation of the HNK-1 epitope (middle column: B, E, H, K. N, Q, T, W) and *Sox10* (right column: C, F, I, L, O, R, U, X) were induced by zebrafish FoxD3 (zFoxD3) and xFoxD3, but not by zFoxD1, xFoxD1, xFoxD2, mouse FoxD4, zFoxD5, or AmphiFoxD. Transfected cells were visualized by anti-GFP antibody in adjacent sections of embryos in which GFP-pCAGGS were co-electroporated (left column: A, D, G, J, M, P, S, V). Ectopic expression of the FoxD proteins was induced on the left-hand side of the neural tube. Numbers in the panel show the number of embryos in which marker overexpression was observed as a fraction of the number of embryos examined.

each paralog. Thus, we next sought to determine whether other paralogous genes in the *FoxD* family have paralog-specific functions, *i.e.*, whether paralogs of FoxD3 have NCC differentiation-inducing activity. Kos et al. (2001) and Dottori et al. (2001) reported that overexpression of FoxD3 in chick neural tubes induces the differentiation of ectopic NCCs, as assessed by the expression of the *Sox10* transcription factor gene and the HNK-1 epitope. We first examined whether the overexpression of FoxD3 orthologs from other vertebrate species would exhibit the same activity when overexpressed in chick neural tube at the level of trunk. As shown in Fig. 2A-F, overexpression of xFoxD3 or zebrafish FoxD3 (zFoxD3) caused marked upregulation of the HNK-1 epitope and *Sox10* expression. Thus, FoxD3 orthologs from distant species of vertebrates can induce the production of ectopic NCCs when overexpressed in chick neural tube.

We next examined the activities of other vertebrate FoxD paralogs. As shown in Fig. 2, neither zFoxD1, xFoxD1, xFoxD2, mouse FoxD4 or zFoxD5 upregulated HNK-1 or *Sox10* expression

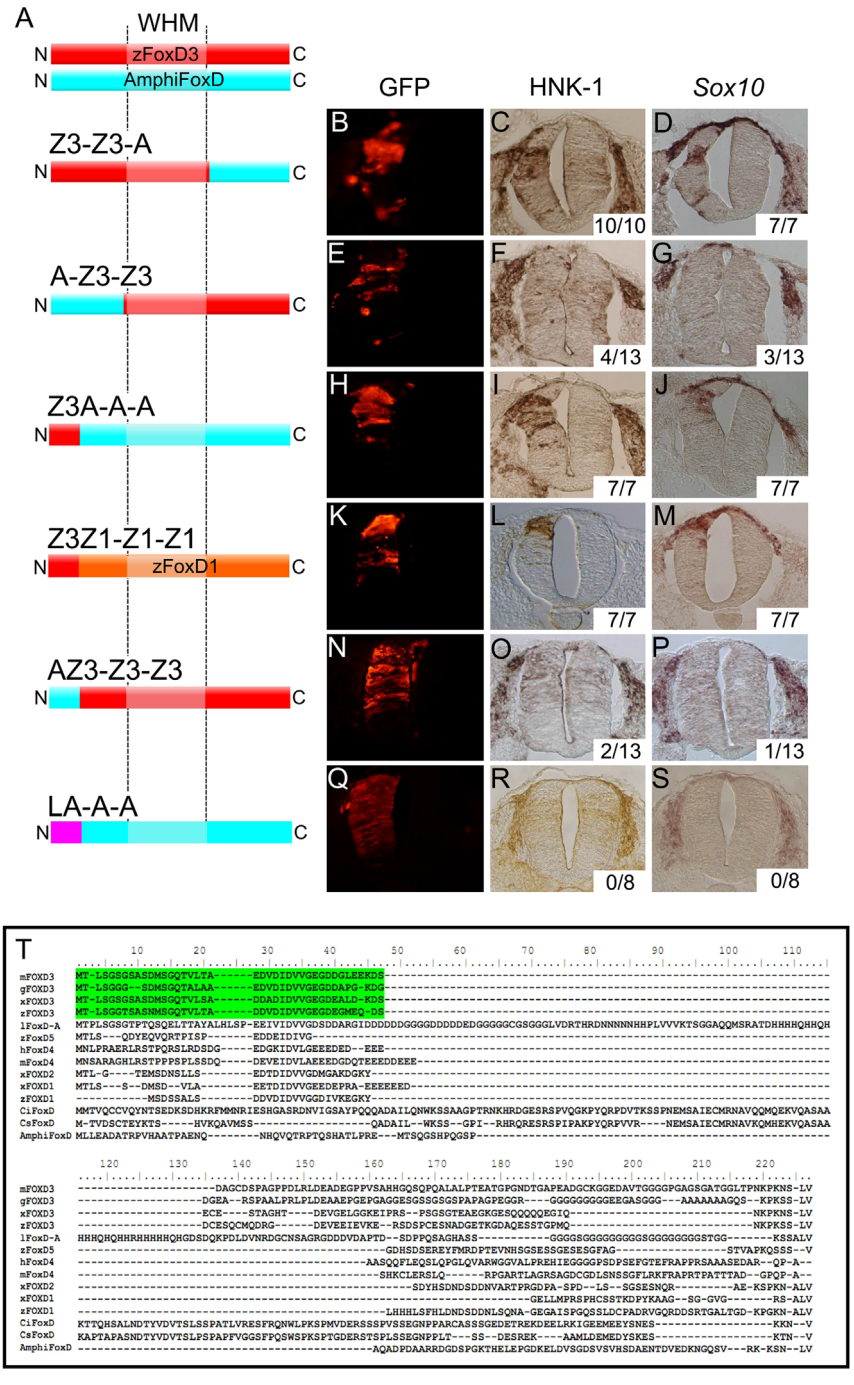

Fig. 3. Effect of overexpression of chimeric FoxD proteins on *Sox10* and HNK-1 epitope expression in chick neural tube. (A) Schematic illustrations of chimeric protein constructs, where amino acid segments from zFoxD3, AmphiFoxD, zFoxD1 and lamprey FoxD-A are shown in red, blue, orange and magenta respectively. Upregulation of the HNK-1 epitope (middle column: C, F, I, L, O, R) and *Sox10* (right column: D, G, J, M, P, S) were induced by chimeric constructs: Z3-Z3-A, Z3A-A-A and Z3Z1-Z1-Z1, but only fairly induced by A-Z3-Z3, AZ3-Z3-Z3 or LA-A-A. Transfected cells were visualized by anti-GFP antibody in adjacent sections of embryos in which GFP-pCAGGS were co-electroporated (left column: B, E, H, K. N, Q). Ectopic expression of the FoxD proteins was induced on the left-hand side of the neural tube. Numbers in the panel show the number of embryos in which marker overexpression was observed as a fraction of the number of embryos examined. (T) Amino acid sequence alignment of the N-terminal portions of proteins encoded by genes of the *FoxD* family. The 39-aa N-terminal segment conserved in FoxD3 genes is shaded green.

when overexpressed in chick neural tubes (Fig. 2G–U). Referring to the phylogeny of the *FoxD* gene family (Yu et al., 2002), we surmised that the sequence motif for ectopic induction of NCCs became fixed only in FoxD3 orthologs after the vertebrate genome duplications. In support of this conclusion, the overexpression of AmphiFoxD also failed to induce any upregulation of HNK-1 or *Sox10* expression (Fig. 2V–X).

The N-terminal sequence of FoxD3 is critical for NCC induction

The amino acid sequence of the DNA-binding, winged-helix motif (WHM) of FoxD3 is highly conserved; only one amino acid substitution is specific to the FoxD3 paralogs (Supplementary Fig. 7). Thus, differences in the sequence outside of WHM are likely to be responsible for specialization of FoxD3 paralog functions. Therefore, to identify the amino acid sequence motif of FoxD3 responsible for NCC induction, we tested the activity of two chimeric proteins in which the portion of zFoxD3 N-terminal or C-terminal to the WHM was replaced with the corresponding portion of AmphiFoxD. The chimera Z3-Z3-A contains the zFoxD3 (Z) sequence N-terminal to the WHM, the zFoxD3 (Z) WHM, and the AmphiFoxD (A) sequence C-terminal to the WHM. The inverse chimera A-Z3-Z3 contains the AmphiFoxD (A) sequence N-terminal to the WHM, the zFoxD3 (Z) WHM, and the zFoxD3 (Z) sequence C-terminal to the WHM (Fig. 3A). We found that the overexpression of the Z3-Z3-A FoxD3 chimera in chick neural tube induced the differentiation of ectopic NCCs, as shown by marked upregulation of HNK-1 and *Sox10* expression (Fig. 3B–D); in contrast, the A-Z3-Z3 FoxD3 chimera failed to show significant NCC inducing activity (Fig. 3E-G). Although some A-Z3-Z3 embryos did have a small number of ectopic NCCs, the induction activity was rather low relative to that of zFoxD3. Thus, we concluded that the portion of the protein N-terminal to the WHM is critical for the NCC differentiation-inducing activity of FoxD3.

An amino acid sequence alignment of the N-terminal portion of FoxD proteins revealed that N-terminus is conserved in FoxD3 but not in other vertebrate paralogs or in amphioxus FoxD (Fig. 3T), suggesting that this conserved region might be important for FoxD3 function. To examine this hypothesis, we produced a chimeric FoxD protein in which the N-terminal 39 amino acids of AmphiFoxD were replaced with the corresponding amino acids of zFoxD3. This modified AmphiFoxD protein (designated Z3A-A-A) induced differentiation of ectopic NCCs when overexpressed in chick neural tube (Fig. 3H–J), confirming that evolutionary changes in the N-terminal 39 amino acids would have been sufficient to confer NCC differentiation-inducing activity on the ancestral FoxD transcription factor. Similarly, zFoxD1 protein in which the N-terminal 39 amino acids were replaced with those of zFoxD3 (Z3Z1-Z1-Z1) also induced differentiation of ectopic NCCs (Fig. 3K–M). On the other hand, zFoxD3 whose N-terminal 39 amino acids were replaced with those from AmphiFoxD (AZ3-Z3-Z3) scarcely induced ectopic NCCs (Fig. 3N–P). Thus, N-terminal 39 amino acids are necessary for FoxD3 to induce NCC differentiation. Searches against the NCBI (http://www.ncbi.nlm.nih.gov/guide/proteins/) and PFam protein databases (http://pfam.sanger.ac.uk/) yielded no proteins other than FoxD proteins containing sequences similar to the N-terminal 39-aa sequence of zFoxD3.

We then asked when the conserved N-terminal sequence was fixed in chordate evolution. FoxD from ascidian *Ciona* shows expression in melanocytes and endodermal cells (Imai et al., 2002; Abitua et al., 2012). *Ciona* FoxD has a highly divergent sequence in N-terminal region, and no conservation observed (Fig. 3T). Thus, the fixation of the N-terminal sequence is likely to have occurred after the divergence of vertebrates from invertebrate chordates. Lamprey was reported to possess a FoxD family gene (*FoxD-A*) that is expressed during neural crest differentiation (Sauka-Spengler et al., 2007). The N-terminal sequence of lamprey FoxD-A is moderately conserved with those of other vertebrate FoxD paralogues (Fig. 3T). We tested the activity of the N-terminal sequence of the lamprey FoxD-A by a fusion construct with AmphiFoxD (Supplementary Fig. 6), and found that the lamprey N-terminal sequence do not provide HNK-1/Sox10 inducing activity to amphioxus FoxD (Fig. 3Q–S). Therefore, lamprey FoxD-A may not be able to substitute for the role of gnathostome FoxD3 in the context of chick neural tube.

Discussion

Neofunctionalization of transcription factors

If evolutionary innovations in animal morphology have arisen largely through co-option of toolkit genes and changes in *cis*-regulatory regions of transcription factors (Carroll et al., 2001; Davidson, 2006), what happens when the transcription factors themselves acquire new functional domains? A new functional domain might allow continued regulation of the transcription factor's ancestral targets while also conferring upon the transcription factor the ability to regulate new target proteins. A new interface for interacting with other transcription factors might be essential for this new function.

Our findings suggest that vertebrate transcription factor FoxD3 underwent this type of molecular evolution during the acquisition of its novel ability to induce NCC differentiation. On the one hand, amphioxus and vertebrate FoxD cognates function similarly, acting as transcriptional repressors that bind to similar DNA sequences. FoxD3 is known to work primarily as a transcriptional repressor via a Groucho-like repressor-interaction motif in its C-terminal domain (Sutton et al., 1996; Pohl and Knöchel, 2001; Sasai et al., 2001; Steiner et al., 2006; Yaklichkin et al., 2007; but note that in some context, it was suggested that vertebrate FoxD3 functions as a transcriptional activator; *e.g.*, Liu and Labosky, 2008). This motif is required for FoxD3 to induce the differentiation of dorsal mesoderm in *Xenopus* embryos (Yaklichkin et al., 2007) and is conserved in AmphiFoxD, consistent with the idea that it is required for *FoxD* genes to play their ancestral role in mesoderm development. On the other hand, the NCC differentiation-inducing function of FoxD3 is unique to vertebrates, and has arisen through the fixation of a specific N-terminal amino acid sequence not present in AmphiFoxD or *Ciona* FoxD. We found that, although lamprey possess migratory neural crest cells, the N-terminal sequence of the lamprey FoxD-A did not provide HNK-1/Sox10-inducing activity when fused with AmphiFoxD. This observation may reflect the variation in the distal part of the lamprey neural crest gene regulatory network compared with that in gnathostomes (Sauka-Spengler et al., 2007; Nikitina and Bronner-Fraser, 2009). In the lamprey embryo, several neural crest specifier genes including *c-Myc*, *Id*, *AP2* and *Snail* are deployed earlier than *FoxD3* and *SoxE* family genes, suggesting that the regulatory linkages among lamprey neural crest specifier genes might be slightly different. Alternatively, this lack of activity may simply be due to technical issues; *i.e.*, N-terminal portion of the lamprey FoxD-A may perform the same role during neural crest differentiation, but just cannot work in the cellular context of the chick neural tube, possibly due to the divergence of the amino acid sequence in the counterpart proteins. In either case, this N-terminal amino acid sequence must constitute a new interface critical for FoxD3 to function in the GRN of NCC differentiation.

Thomas and Erickson (2009) indicated that FoxD3 represses Mitf expression in avian neural crest cells, and thus suppress neural crest cells from differentiation into pigment cells. This effect of FoxD3 on Mitf expression is not dependent on the DNA

binding, but on sequestration of Pax3. Abitua et al. (2012) showed that ascidian FoxD also suppresses Mitf expression. Moreover, they indicated that its portion N-terminal to WHM is sufficient for this suppression. These studies may suggest that the N-terminal sequence unique to vertebrate FoxD3 may be involved in the interaction with Pax3 or other transcription factors, and those interactions may confer the new functions of FoxD3 protein in vertebrate neural crest development.

Evolution of the neural crest GRN

For those interested in the evolutionary origin of vertebrates, an understanding of the evolution of the neural crest GRN is critical. That the neural crest regulatory genes can be divided into neural plate border specifiers and NCC specifiers illuminates the stepwise evolution of the neural crest GRN. Because protochordate neural plate border specifiers, like those of vertebrates, are expressed in the corresponding region between the neural and non-neural ectoderm (Meulemans and Bronner-Fraser, 2004; Yu et al., 2008; Yu, 2010), their eventual involvement in NCC differentiation would not require a change in their expression patterns. Thus, as the first step in the evolution of the neural crest GRN, the border specifiers have to recruit a set of genes (neural crest specifiers) as their downstream targets. These genes may not have been recruited simultaneously. Duplicate paralogs of *SoxE*, *snail/slug*, and *AP-2* are expressed in NCCs, indicating that recruitment of these genes to the neural crest GRN occurred before the genome duplications (Wada and Makabe, 2006). In contrast, among the five known vertebrate FoxD paralogs, only FoxD3 is expressed in the neural crest (Yu et al., 2002,2004; Wada and Makabe, 2006). Therefore, FoxD3 might have been recruited slightly later than the other neural crest specifiers, after the genome duplications.

The second step in the evolution of the neural crest GRN might be the acquisition of target effector genes, such as cadherin and collagen genes, for the neural crest specifiers. Interestingly, these effector genes appear to have been present during the vertebrate genome duplications but, in several cases, only certain paralogs were recruited as neural crest effectors (*e.g.*, cadherin6, cadherin7, col2a1, and rhoB), suggesting that neofunctionalization of some effectors to NCC development occurred after the genome duplications (Wada and Makabe, 2006). Actually, cadherin7 was suggested as direct FoxD3 target (Dottori et al. 2001). Therefore, the neural crest GRN may have been completed by the recruitment of some novel target genes after the genome duplications.

During its evolution, the neural crest GRN must have gained several new regulatory interactions, probably through the acquisition of new *cis*-regulatory regions by target genes (Yu et al., 2008). In addition, because most of the transcription factor genes in the neural crest GRN function not only in NCCs but also in other cells, interactions between transcription factors may be essential for NCC-specific regulation of target gene expression. Our FoxD fusion construct studies have shown that the N-terminal region of FoxD3 is critical for its role in neural crest development. SoxE, on the other hand, may not have a fixed motif specific to neural crest development, because *Drosophila* SoxE can substitute functionally for vertebrate SoxE in NCC differentiation (Cossais et al., 2010). Examination of the neural crest GRN from the aspect of interactions between transcription factors may shed new light on neural crest evolution, and will provide more general insights into how novel GRNs emerged during evolution.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.11.010.

References

Abitua, P.B., Wagner, E., Navarrete, I.A., Levine, M., 2012. Identification of a rudimentary neural crest in a non-vertebrate chordate. Nature 492, 104–107.
Aniello, F., Locascio, A., Villani, M.G., Gregorio, A.D., Fucci, L., Branno, M., 1999. Identification and developmental expression of Ci-msxb: a novel homologue of *Drosophila msh* gene in *Ciona intestinalis*. Mech. Dev. 88, 123–126.
Bourlat, S.J., et al., 2006. Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. Nature 444, 85–88.
Caracciolo, A., Gregorio, A.D., Aniello, F., Lauro, R.D., Branno, M., 2000. Identification and developmental expression of three Distal-less homeobox containing genes in the ascidian *Ciona intestinalis*. Mech. Dev. 99, 173–176.
Carroll, S.B., Greiner, J.K., Weatherbee, S.D., 2001. From DNA to diversity. Blackwell, Malden.
Cossais, F., Sock, E., Horning, J., Schreiner, S., Kellerer, S., Bösl, M.R., Russel, S., Wegner, M., 2010. Replacement of mouse Sox10 by the Drosophila ortholog Sox100B provides evidence for co-option of SoxE proteins into vertebrate-specific gene-regulatory networks through altered expression. Dev. Biol. 341, 267–281.
Czerny, T., Busslinger, M., 1995. DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). Mol. Cell Biol. 15, 2858–2871.
Davidson, E.H., 2006. The Regulatory Genome. Academic Press, San Diego.
Dottori, M., Gross, M.K., Labosky, P., Goulding, M., 2001. The winged-helix transcription factor Foxd3 supresses interneuron differentiation and promotes neural crest cell fate. Development 128, 4127–4138.
Fisher, A.L., Caudy, M., 1998. Groucho proteins: transcriptional corepressors for specific subsets of DNA binding transcription factors in vertebrates and invertebrates. Genes Dev. 12, 1931–1940.
Galant, R., Carroll, S.B., 2002. Evolution of a transcriptional repression domain in an insect Hox protein. Nature 415, 910–913.
Gans, C., Northcutt, R.G., 1983. Neural crest and the origin of vertebrates: a new head. Science 220, 268–274.
Gostling, N.J., Shimeld, S.M., 2003. Protochordate Zic genes define primitive somite compartments and highlight molecular changes underlying neural crest evolution. Evol. Dev. 5, 136–144.
Hilger-Eversheim, K., Moser, M., Schorle, H., Buettner, R., 2000. Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. Gene 260, 1–12.
Holland, L.Z., Schubert, M., Kozmik, Z., Holland, N.D., 1999. AmphiPax3/7, an amphioxus paired box gene: insights into chordate myogenesis, neurogenesis, and the possible evolutionary precursor of definitive vertebrate neural crest. Evo. Dev. 1, 153–165.
Holland, N.D., Panganiban, G., Henyey, E.L., Holland, L.Z., 1996. Sequence and developmental expression of *AmphiDll*, an amphioxus *Distal-less* gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of craniate forebrain and neural crest. Development 122, 2911–2920.
Hong, C.-S., Saint-Jeannet, J.-P., 2005. Sox proteins and neural crest development. Sem. Dev. Biol., 16.
Imai, K.S., Satoh, N., Satou, Y., 2002. An essential role of FoxD gene in notochord induction in *Ciona* embryos. Development 129, 3441–3453.
Jennings, B.H., Pickles, L.M., Wainright, S.M., Roe, S.M., Pearl, L.H., Ish-Holowicz, D., 2006. Molecular recognition of transcriptional repressor motifs by the WD domain of the Groucho/TLE corepressor. Mol. Cell 22, 645–655.
Jin, C., Marsden, I., Chen, X., Liao, X., 1999. Dynamic DNA contacts observed in the NMR structure of winged helix protein–DNA complex. J. Mol. Biol. 289, 683–690.
Kawashima, T., Kawashima, S., Tanaka, C., Murai, M., Yoneda, M., Putnam, N.H., Rokhsar, D.S., Kanehisa, M., Satoh, N., Wada, H., 2009. Domain shuffling and the evolution of vertebrates. Genome Res. 19, 1393–1403.
Kos, R., Reedy, M.V., Johnson, R.L., Erickson, C.A., 2001. The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development 128, 1467–1479.
Kozmik, Z., Daube, M., Frei, E., Norman, B., Kos, L., Dishaw, L.J., Noll, M., Piatigorsky, J., 2003. Role of Pax genes in eye evolution: a cnidarian PaxB gene uniting Pax2 and Pax6 functions. Dev. Cell 5, 773–785.

Langeland, J., Tomsa, J.M., Jackman, W.R., Kimmel, C.B., 1998. An amphioxus snail gene: expression in paraxial mesoderm and neural plate suggests a conserved role in patterning the chordate embryo. Dev. Genes Evol. 208, 569–577.
Linker, C., Bronner-Fraser, M., Mayor, R., 2000. Relationship between gene expression domains of Xsnail, Xslug, and Xtwist and cell movement in the prospective neural crest of Xenopus. Dev. Biol. 224, 215–225.
Liu, Y., Labosky, P.A., 2008. Regulation of embryonic stem cell self-renewal and pluripotency by Foxd3. Stem Cells 26, 2475–2484.
Lynch, V.J., Tanzer, A., Wang, Y., Leung, F.C., Gellersen, B., Emera, D., Wagner, G.P., 2008. Adaptive changes in the transcription factor HoxA-11 are essential for the evolution of pregnancy in mammals. Proc. Natl. Acad. Sci. U.S.A. 105, 14928–14933.
Meulemans, D., Bronner-Fraser, M., 2002. Amphioxus and lamprey AP-2 genes: implications for neural crest evolution and migration pattern. Development 129, 4953–4962.
Meulemans, D., Bronner-Fraser, M., 2004. Gene-regulatory interactions in neural crest evolution and development. Dev. Cell 7, 291–299.
Meulemans, D., Bronner-Fraser, M., 2005. Central role of gene cooption in neural crest evolution. J. Exp. Zool. B Mol. Dev. Evol. 304, 298–303.
Meulemans, D., Bronner-Fraser, M., 2007. Insights from amphioxus into the evolution of vertebrate cartilage. PLoS ONE 2, e787.
Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K., Yasuda, K., 1999. Efficient targeting of gene expression in chick embryos by microelectroporation. Dev. Growth Differ. 41, 335–344.
Nikitina, N.V., Bronner-Fraser, M., 2009. Gene regulatory networks that control the specification of neural-crest cells in the lamprey. Biochem. Biophys. Acta 1789, 274–278.
Pierrou, S., Hellqvist, M., Samuelsson, L., Enerbäck, S., Carlsson, P., 1994. Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. EMBO J. 13, 5002–5012.
Pohl, B.S., Knöchel, W., 2001. Overexpression of the transcriptional repressor FoxD3 prevents neural crest formation in Xenopus embryos. Mech. Dev. 103, 93–106.
Putnam, N.H., et al., 2008. The amphioxus genome and the evolution of the chordate karyotype. Nature 453, 1064–1071.
Ronshaugen, M., McGinnis, N., McGinnis, W., 2002. Hox protein mutation and macroevolution of the insect body plan. Nature 415, 914–917.
Saleem, R.A., Banerjee-Basu, S., Berry, F.B., Baxevanis, A.D., Walter, M.A., 2001. Analyses of the effects that disease-causing missense mutations have on the structure and function of the winged-helix protein FOXC1. Am. J. Hum. Genet. 68, 627–641.
Sasai, N., Mizuseki, K., Sasai, Y., 2001. Requirement of FoxD3-class signaling for neural crest determination in *Xenopus*. Development 128, 2525–2536.
Sauka-Spengler, T., Meulemans, D., Jones, M., Bronner-Fraser, M., 2007. Ancient evolutionary origin of the neural crest gene regulatory network. Dev. Cell 13, 405–420.
Sharman, A.C., Shimeld, S.M., Holland, P.W.H., 1999. An amphioxus Msx gene expressed predominantly in the dorsal neural tube. Dev. Genes Evol. 209, 260–263.
Shiga, Y., Yasumoto, R., Yamagata, H., Hayashi, S., 2002. Evolving role of antennapedia protein in arthropod limb patterning. Development 129, 3555–3561.
Steiner, A.B., Engleka, M.J., Lu, Q., Piwarzyk, E.C., Yaklinchkin, S., Lefebvre, J.L., Walters, J.W., Pineda-Salgado, L., Labosky, P.A., Kessler, D.S., 2006. FoxD3 regulation of nodal in Spemann organizer is essential xenopus dorsal mesoderm development. Development 133, 4827–4838.
Sutton, J., Costa, R., Klung, M., Field, L., Xu, D., Largaespada, D.A., Fletcher, C.F., Jenkins, N.A., Copeland, N.G., Klemsz, M., Hromas, R., 1996. *Genesis*, a winged helix transcriptional repressor with expression restricted to embryonic stem cells. J. Biol. Chem. 271, 23126–23133.
Thomas, A.J., Erickson, C.A., 2009. FOXD3 regulates the lineage switch between neural crest-derived glial cells and pigment cells by repressing MITF through a non-canonical mechanism. Development 136, 1849–1858.
Tucker, G.C., Delarue, M., Zada, S., Boucaut, J.C., Thiery, J.P., 1988. Expression of the HNK-1/NC-1 epitope in early vertebrate neurogenesis. Cell Tissue Res. 251, 457–465.
Wada, H., 2010. Origin and genetic evolution of the vertebrate skeleton. Zool. Sci. 27, 119–123.
Wada, H., Makabe, K., 2006. Genome duplications of early vertebrates as a possible chronicle of the evolutionary history of the neural crest. Int. J. Bio. Sci 2, 133–141.
Wada, H., Holland, P.W.H., Sato, S., Yamamoto, H., Satoh, N., 1997. Neural tube is partially dorsalized by overexpression of *HrPax-37*: the ascidian homologue of *Pax-3* and *Pax-7*. Dev. Biol. 187, 240–252.
Wada, H., Escriva, H., Zhang, S., Laudet, V., 2006. Conserved RARE localoization in amphioxus *Hox* clusters and implications for *Hox* code evolution in the vertebrate neural crest. Dev. Dyn. 235, 1522–1531.
Wu, S.C., Grindley, J., Winnier, G.E., Hargett, L., Hogan, B.L., 1998. Mouse Mesenchyme forkhead 2 (Mf2): expression, DNA binding and induction by sonic hedgehog during somitogenesis. Mech. Dev. 70, 3–13.
Yaklichkin, S., Steiner, A.B., Lu, Q., Kessler, D.S., 2007. FoxD3 and Grg4 physically interact to repress transcription and induce mesoderm in *Xenopus*. J. Biol. Chem. 282, 2548–2557.
Yu, J.K., Holland, N.D., Holland, L.Z., 2002. An amphioxus winged helix/forkhea gene, AmphiFoxD: insights into vertebrate neural crest evolution. Dev. Dyn. 225, 289–297.
Yu, J.K., Holland, N.D., Holland, L.Z., 2004. Tissue-specific expression of FoxD reporter constructs in amphioxus embryos. Dev. Biol. 274, 452–461.
Yu, J.K., Meulemans, D., McKeown, S.J., Bronner-Fraser, M., 2008. Insights from the amphioxus genome on the origin of vertebrate neural crest. Genome Res. 18, 1127–1132.
Yu, J.K.S., 2010. The evolutionary origin of the vertebrate neural crest and its developmental gene regulatory network – insights from amphioxus. Zoology 113, 1–9.